The Rapidly Acting Antidepressant Ketamine and the mGlu2/3 Receptor Antagonist LY341495 Rapidly Engage Dopaminergic Mood Circuits


Received March 18, 2016; accepted May 10, 2016

ABSTRACT

Ketamine is a rapidly acting antidepressant in patients with treatment-resistant depression (TRD). Although the mechanisms underlying these effects are not fully established, inquiry to date has focused on the triggering of synaptogenesis transduction pathways via glutamatergic mechanisms. Preclinical data suggest that blockade of metabotropic glutamate (mGlu2/3) receptors shares many overlapping features and mechanisms with ketamine and may also provide rapid efficacy for TRD patients. Central dopamine circuitry is recognized as an end target for mood regulation and hedonic valuation and yet has been largely neglected in mechanistic studies of antidepressant-relevant effects of ketamine. Herein, we evaluated the changes in dopaminergic neurotransmission after acute administration of ketamine and the mGlu2/3 receptor antagonist LY341495 [(2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid] in preclinical models using electrophysiologic, neurochemical, and behavioral endpoints. When given acutely, both ketamine and LY341495, but not the selective serotonin reuptake inhibitor (SSRI) citalopram, increased the number of spontaneously active dopamine neurons in the ventral tegmental area (VTA), increased extracellular levels of dopamine in the nucleus accumbens and prefrontal cortex, and enhanced the locomotor stimulatory effects of the dopamine D2/3 receptor agonist quinpirole. Further, both ketamine and LY341495 reduced immobility time in the tail-suspension assay in CD1 mice, which are relatively resistant to SSRI antidepressants. Both the VTA neuronal activation and the antidepressant phenotype induced by ketamine and LY341495 were attenuated by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo- (9CI)-benzo[f]quinoxaline-7-sulfonamide, indicating AMPA-dependent effects. These findings provide another overlapping mechanism of action of ketamine and mGlu2/3 receptor antagonism that differentiates them from conventional antidepressants and thus support the potential rapidly acting antidepressant actions of mGlu2/3 receptor antagonism in patients.

Introduction

Although the monoamine hypothesis of depression has been the prevailing foundational principle underlying our understanding of the clinical findings with antidepressants since the 1950s, there are two principal gaps. First, not all patients respond to monoamine-based antidepressants (Rush et al., 2006). Second, full-blown antidepressant efficacy is not achieved after acute dosing of these conventional antidepressants, despite their immediate impact on central monoamines (Iversen, 2005; Millan, 2009). In contrast, ketamine produces symptom relief in several populations of depressed patients that begins almost immediately and can last for several weeks (Abdallah et al., 2015).

Most strikingly, ketamine engenders these transformative behavioral and mood changes in patients who have not responded to multiple standard-of-care antidepressants. This clinical response was predicted from preclinical data (Trullas and Skolnick, 1990) and established in patients by Berman and colleagues (Berman et al., 2000). With subsequent verification in multiple clinical studies, and extension to other NMDA receptor antagonists (cf. Zarate et al., 2006; Abdallah et al., 2015), research into the mechanisms of action of antidepressant effects has been markedly energized (Sanacora and Schatzberg, 2009). Data generated over the past several years have identified key biologic targets underlying the antidepressant-related actions of ketamine. Collectively, these data point to the initiation of a biologic cascade, beginning with the enhancement of synaptic levels of glutamate via inhibition of fast-spiking GABAergic interneurons (Homayoun and Moghaddam, 2007). The current model, built primarily on behavioral, biochemical, electrophysiologic, and neurochemical studies, suggests that ketamine acts on glutamatergic inputs to the ventral tegmental area (VTA) and accumbens to increase neuronal activity in the VTA and accumbens, leading to the enhancement of dopamine release and activity in the prefrontal cortex and limbic regions. This increases dopamine activity in the prefrontal cortex and limbic regions, leading to the behavioral and mood changes seen in depressed patients.

Studies were sponsored and funded by Eli Lilly and Co., Indianapolis, Indiana. All authors were employees of, and stockholders in, Eli Lilly and Company at the time the experiments were conducted.

dx.doi.org/10.1124/jpet.116.233627.

ABBREVIATIONS: aCSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, analysis of variance; BAS, BioAnalytical Systems; GABA, γ-aminobutyric acid; HPLC, high-performance liquid chromatography; LTP, long-term potentiation; LY341495, (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; mGlu, metabotropic glutamate; NAC, nucleus accumbens; NBOX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-(9CI)-benzo[f]quinoxaline-7-sulfonamide; NIH, National Institutes of Health; SSRI, selective serotonin reuptake inhibitor; TRD, treatment-resistant depression; VTA, ventral tegmental area.
and morphologic evidence in rodent models, posits a rapid engagement of synaptic plasticity triggered by the facilitation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function, leading to enhanced signaling through mammalian target of rapamycin pathways, thus stimulating synaptogenesis. These morphologic changes are associated with improvement in synaptic function and reduced depression-related behaviors (Li et al., 2010; Duman et al., 2012; Abdallah et al., 2015; Miller et al., 2016). Similar to ketamine, metabotropic glutamate (mGlu) 2/3 receptor antagonists, such as LY341495 [(2S)-2-amino-2-[(1S,2S)-2-carboxycyclopent-1-yl]-3-(xanth-9-yl)-propanoic acid] (Kingston et al., 1998), have also been reported to engage these core biochemical, morphologic, and behavioral processes (Hascup et al., 2010; Koike et al., 2011; Dwyer et al., 2012). Recently, a profound and enduring (6 month follow-up) impact of psilocybin in depressed patients has also been uncovered (R.L. Carhart-Harris, personal communication), the biologic basis of which has yet to be explored.

To date, almost no focus has been directed toward dopamine pathways as they might relate to the antidepressant efficacy of ketamine (Abdallah et al., 2015). Yet, ketamine and other NMDA receptor channel blockers are well characterized facilitators of dopamine neurotransmission (Moghaddam and Krystal, 2012). Furthermore, the pioneering work of Fibiger and colleagues documented that the antidepressant efficacy of monoamine-based antidepressants was associated with a sensitization of dopamine outflow and function (Phillips and Fibiger, 1976; Fibiger and Phillips, 1981; Syparaki and Fibiger, 1981). Chronic administration of antidepressants or nonpharmacologic treatments of depression (e.g., electroconvulsive shock, sleep deprivation) sensitizes mesolimbic dopamine pathways (Tufik et al., 1978; D’Aquila et al., 1997; Willner, 1997; West and Weiss, 2011) that are key components of established mood-related circuitry (Price and Drevets, 2010).

Importantly, these data have led researchers to specifically target dopamine augmentation in the discovery of novel antidepressants (Skolnick and Basile, 2007). Indeed, it has been hypothesized that the augmented levels of dopamine in the medial prefrontal cortex induced by combinations of olanzapine and fluoxetine (Symbax), the only approved medication for TRD, might account for its efficacy in TRD patients (Zhang et al., 2000; Koch et al., 2004). Optogenetic studies in mice have provided increased focus on the role of midbrain dopamine neurons in depression (Chaudhury et al., 2013; Tye et al., 2013). Grace and colleagues demonstrated that ketamine reversed the suppression of ventral tegmental area (VTA) dopamine neuron activity associated with learned helplessness behaviors and restored hippocampal long-term potentiation (LTP), an effect mediated by D1 receptors in the nucleus accumbens (NAc) (Belujon and Grace, 2014). Together, the data suggest that changes in dopamine neurotransmission might be a critical component of the antidepressant effects of ketamine.

The present set of experiments was initiated to evaluate changes in dopaminergic activity and function after acutely administered ketamine. Since mGlu2/3 receptor antagonists share biologic actions with ketamine, as discussed herein already, we provide a parallel investigation with the mGlu2/3 receptor antagonist LY341495. Citalopram was used as a conventional selective serotonin reuptake inhibitor (SSRI) antidepressant control drug. We hypothesized that ketamine and LY341495 would rapidly (after a single dose) engage dopamine neurons in the VTA and facilitate dopamine-mediated behaviors, whereas citalopram would not. Since AMPA receptor facilitation is thought to be an upstream regulator of antidepressant-related biochemistry and behavior (Alt et al., 2006; Abdallah et al., 2015), we further hypothesized that facilitation of dopamine neuronal activity and dopamine-mediated behaviors by ketamine and LY341495 would be blocked by the AMPA receptor antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-(9CI)-benzofl quinoxaline-7-sulfonamide (NBQX) and LY341495. The results of the present series of studies confirmed these hypotheses and thus link the downstream mechanisms of ketamine to those engendered by conventional antidepressants that arise only after multiple weeks of dosing.

Materials and Methods

Animals. Male National Institutes of Health (NIH)-Swiss (weight, 24–35 g) (Harlan Laboratories, Indianapolis, IN), Balb/C mice (weight, 20–30 g) (Harlan Sprague-Dawley, Indianapolis, IN), and CD-1 mice (weight, 25–30 g) (Charles River Breeder, Charles River Laboratories International, Inc., Wilmington, MA) were used. Mice were housed in plastic cages (40.6 × 20.3 × 15.2 cm) with 10–12 mice/cage. Electrophysiologic and in vivo microdialysis experiments in the nucleus accumbens were conducted in male Sprague-Dawley rats (Taconic Farms, Inc., Albany, NY) with body weights of 250–330 g. Male Wistar rats (weight, 308–398 g) from Charles River were used in frontal cortex microdialysis studies. Rats were housed in groups of five per cage in standard rat husbandry cages (40.6 × 20.3 × 15.2 cm) for behavioral studies and in groups of two to four for electrophysiologic and neurochemical studies.

Rats and mice were housed in separate colony rooms, and only male animals were used throughout. All animals were acclimated to the vivarium for a minimum of 72 hours before each experiment. Water and rodent chow were available ad libitum except during the test procedure. The vivarium was illuminated from 06:00 to 18:00, and experiments were conducted between the hours of 10:00 and 16:00. Animals were transported from the vivarium to the testing area in their home cages and allowed to adapt to the new environment for at least 1 hour before behavioral testing. All animals were experimentally and drug-naive at the time of testing and were used for only one experiment. All experiments were conducted according to the NIH Guidelines for Care and Use of Laboratory Animals under protocols approved by the local institutional animal care and use committee.

Compounds. LY341495 (Kingston et al., 1998) was synthesized at Lilly Research Laboratories (Indianapolis, IN). Citalopram HBr and imipramine HCl were purchased from Sigma-Aldrich (St. Louis, MO), and (±)-ketamine HCl, racemic ketamine, (±)-quinpirole HCl, and NBQX were purchased from Tocris Biosciences (Ellisville, MO). LY341495 and NBQX were dissolved in sterile water and titrated to solution with dilute NaOH (titrated to pH 7-8 using dilute lactic acid). (±)-Ketamine was dissolved in sterile water; in some experiments (noted in Materials and Methods), S-(+) -ketamine was used. Citalopram and imipramine were dissolved in 0.9% NaCl. All compounds and vehicle were administered to mice in a volume of 10 ml/kg or rats in a volume of 1 ml/kg. LY341495 and citalopram were dosed by the i.p. route unless otherwise noted. (±)-Ketamine was administered by i.v. injection in the electrophysiology and behavioral studies in rats and i.p. in mouse experiments and by i.p. injection in the nucleus accumbens studies. (±)-Ketamine was dosed s.c. in the prefrontal cortex microdialysis experiments.

In Vivo Electrophysiology. Male rats were anesthetized using chloral hydrate (400 mg/kg, i.p.), followed by supplemental doses as needed to maintain anesthesia. Rats were placed on a heating pad to maintain body temperature at 37°C throughout the procedures. An i.v. catheter, consisting of PE10 tubing (Becton Dickinson, Sparks, MD) connected to a syringe via 30-gauge hypodermic needle (1 inch long; Exel International, Los Angeles, CA), was placed in the jugular vein.
for administration of test compounds and supplemental anesthesia. The catheter was secured into the vein using suture thread (3-0 Black Braided Silk; Roboz Surgical, Rockville, MD), tied tightly enough to prevent slippage but not so tight as to constrict blood flow. After catheter implantation, rats were positioned tightly into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), a hole was drilled in the skull overlying the targeted VTA coordinates (anteroposterior: −5.3 to −5.7 mm, mediolateral: +0.5 to −0.9 mm; dorsventral: −6.0 to −8.5 mm, relative to bregma), and the dura was carefully resected. A single-barrel micropipette (Radnoti Starbore capillary tube, 1.8-mm O.D.; pulled using a Narishige PE-2 vertical puller; broken back to a tip diameter ~2–6 μm, and filled with 2M NaCl solution; impedance 3–10 MΩ) was mounted into a Burleigh Inchworm 8200 microdrive for single-unit extracellular recordings.

Electrophysiologic signals were amplified by a Dagan 2400 preamplifier (low-cut 300 Hz, high-cut 3000 Hz; gain 1×). Data were recorded and analyzed using a Micro1401 data acquisition system with Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, UK). Electrophysiologic properties of spontaneously active VTA dopamine cells were characterized by lowering recording electrodes slowly (3 μm/s) through the dorsoventral extent of the VTA along 9–12 predefined tracks (separated by 200 μm) in a grid pattern throughout the entire nucleus. Dopamine cells were identified and distinguished from nondopamine cells based on their unique long-duration (2.5–4 milliseconds), triphasic waveform and slow irregular firing rate (2–10 spikes/s) (Grace and Bunney, 1983). For each track, the number of spontaneously active dopamine cells, the average firing rate, and the percentage of spikes occurring in bursts were analyzed. A burst was defined as a period of rapid cell firing, with burst initiation identified as an interspike interval < 80 milliseconds between two consecutive action potentials and interspike interval > 160 milliseconds indicating the end of the burst.

For each dose level, spontaneously active dopamine cells were characterized throughout two or three tracks. Doses were administered (i.e., 10-minute pretreatment) in ascending order to achieve a cumulative dose-effect function. Thus, drug vehicle was administered 10 minutes before the first track, 3 mg/kg ketamine was administered 10 minutes before track 4, 10 mg/kg ketamine was administered 10 minutes before track 7, and 17 mg/kg ketamine was administered 10 minutes before track 10. In separate experiments, the mGlu2/3 antagonist, LY341495, and the SSRI, citalopram were tested for their effects on VTA dopamine cells when administered alone or in combination. The AMPA receptor antagonist, NBQX (10 mg/kg, i.v.), was administered 10 minutes before track 7, and 17 mg/kg ketamine was administered 10 minutes before track 10. In separate experiments, the mGlu2/3 antagonist, LY341495, and the SSRI, citalopram were tested for their effects on VTA dopamine cells when administered alone and also for its ability to block the increase in spontaneously active dopamine cells produced by z-ketamine (17 mg/kg, i.p.) or LY341495 (3 mg/kg, i.p.).

In Vivo Microdialysis: Medial Prefrontal Cortex. Microdialysis probes (MAB 4.7 Cu 6kDa cutoff; RoYem Scientific, Ltd., Bedford, UK) were implanted under isoflurane anesthesia into the medial prefrontal cortex using the following coordinates (from bregma and dura surface; probe angled at 12 degrees): AP medial prefrontal cortex using the following coordinates (from bregma −1.5 mm, DV −2.0–2.2 mm; and P: −5.1–5.5 mm, relative to bregma). The probe was inserted while the animal was being held; the probe was glued into the guide so that 2 mm of the probe extended beyond the cannula. The input tube of the dialysis probe was connected to a syringe pump (BeeHive and BabyBee, BAS), which delivered an ACSF solution containing 150 mM NaCl, 3 mM KCl, 1.7 mM CaCl₂, and 0.9 mM MgCl₂ (pH 6.0) at a rate of 0.8 μl/min overnight. The next morning, syringes were refilled with ACSF, and the flow rate was increased to 1.5 μl/min; the output tubes from the rats were attached to a refrigerated fraction collector (Honey-Comb, BAS). After at least three stable baseline samples with <20% variation were obtained, drugs were administered and rats were immediately returned to the microdialysis chambers.

Concentrations of monoamines and metabolites in the dialysates were analyzed simultaneously using an off-line HPLC analytical method. For analysis, dialysate samples were transferred to a refrigerated (AS-700) Eicom Insight autosampler/injector. A BDS-Hypersil 3 μm C18 analytical column (2 × 150 mm from Keystone Scientific, Bellefonte, PA) with a 10-port HPLC valve; a 20-s sample loop was used in configuration with a small sample cleanup column (BDS Hypersil 3 μm C18, 2 × 10 mm), which trapped late-eluting peaks contained in the dialysate samples. The mobile phase for the columns consisted of 75 mM NaPO₄ monobasic, 350 mg/l 1-octanesulfonic acid sodium salt, 0.5 mM EDTA, 0.8% tetrahydrofuran (HPLC grade, inhibitor-free) and 8% acetonitrile in pH 3 (adjusted with phosphoric acid). The flow rate for both columns was 0.22 ml/min and the temperature for the analytical and cleanup columns was 40°C and ambient temperature, respectively. An electrochemical detector with standard configuration, including a DC amperometric flow cell, a single channel graphite electrode, an Ag/AgCl reference electrode and a 25 μM gasket, with dual glassy carbon electrodes, (E₁ = 750 mV,
E2 = 50 mV, range = 0.5 nA and 0.2 nA on the respective electrodes) (Eicom Corp., San Diego, CA) was used for sample analysis. 5-HT and monoamine metabolites were detected at E1 and NE and DA were detected at E2. The data were collected on two channels using an EZChrom chromatography data system (Scientific Software, San Ramon, CA) running on a Compaq computer that calculated peak heights and sample concentrations. The level of sensitivity for NE, DA and 5-HT was 0.1 pmol/ml dialysate or 2 fmol/sample (20 μl).

Data analysis was conducted using GraphPad Prism (San Diego, CA). Each animal served as its own control to establish baseline efflux of the monoamine or metabolite being measured as a percentage of baseline value compared with drug effect. A two-way ANOVA with a Bonferroni’s posttest was conducted. Additionally, the average percentage of baseline postdose was analyzed to create a bar graph, which was analyzed using a one-way ANOVA, followed by Dunnett’s multiple comparison test against vehicle control.

**Potentiation of Quinpirole-Induced Locomotor Activity.**
Sensitization within the dopamine pathways can be measured in a number of ways, one of which is to evaluate the increased sensitivity to the locomotor effects of dopaminergic agonists (Willner, 1997; D’Aquila et al., 2000). A mouse model has been disclosed that was used to evaluate the impact of antidepressant treatments on the behavioral effects of the dopamine D2/3 agonist, quinpirole (Marsteller et al., 2009). Male Balb/C mice were used in these studies. Locomotor activity was measured with a 20-station Photobeam Activity System (San Diego Instruments, San Diego, CA) with seven photocells per station. Animals were weighed, injected with compound i.p., and returned to their home cages for 0.5 hour. They were then injected with quinpirole and placed back into the home cages for an additional 2 hours before placing them into locomotor activity boxes (40.6 × 20.3 × 15.2 cm). Locomotion was then assessed for a 2-hour period immediately after they were placed into the locomotor activity boxes.

Data were collected as total ambulation (where ambulation was defined as the sequential breaking of adjacent photo beams) in 10-minute intervals for the entire period. The mean of the total ambulations for the last hour of the experimental session was summarized. ANOVA with post hoc Dunnett’s test was used to evaluate the dose-response functions separately from drug alone or drug plus quinpirole. Ten mice were used per group and each animal was used only once.

![Graphs](image-url)
**Forced-Swim Test: Rats.** Male Sprague-Dawley rats were brought to the testing room at least 1 hour before testing. Rats were placed in clear plastic cylinders (diameter: 18 cm; height: 40 cm) filled with water (22–25°C) to a depth of 16 cm for 15 minutes. On the next day, the same procedure was followed in the presence of vehicle or drug; immobility time of each rat was recorded for the first 5 minutes of the experiment, and the rats were then removed from the chamber, dried, and warmed. A rat was regarded as immobile when floating motionless or making only those movements necessary to keep its head above the water. (>)-Ketamine was dosed 30 minutes before testing by i.v. injection (tail vein). LY341495 was given i.p., 30 minutes before, NBQX i.p. 60 minutes before, and citalopram p.o. 60 minutes before testing. The data of immobility (in seconds) were analyzed by one-way ANOVA followed by post-hoc Dunnett tests.

**Forced-Swim Test: Mice.** Male NIH Swiss mice were used. This test was performed using the original method described earlier (Porsolt et al., 1977). Briefly, mice were placed individually in clear plastic cylinders (diameter, 10 cm; height, 25 cm) filled to 6 cm with 22–25°C water for 6 minutes. The duration of immobility was recorded during the last 4 minutes of a 6-minute trial. A mouse was regarded as immobile when floating motionless or making only those movements necessary to keep its head above the water. All compounds were administered i.p. 30 minutes before testing except NBQX (60 minutes). Data were analyzed by ANOVA followed by post hoc Dunnett’s tests.

**Tail-Suspension Test in SSRI-Resistant Mice.** Male CD1 mice were used in these studies. An automated tail-suspension apparatus (Med Associates Inc., SOF-821) was used. The tail was secured to a lever in the ceiling of the chamber. The duration of immobility was recorded by a force transducer connected to the lever for a period of 5 minutes. All compounds were administered i.p. 30 minutes before testing.

**Results**

**Electrophysiology.** The hypothesis that ketamine and LY341495 would augment dopaminergic tone emanating from the dopamine cell bodies of the VTA was tested here and compared with the effects of the SSRI antidepressant citalopram. (>)-Ketamine significantly increased the number of spontaneously active dopamine cells in the VTA (Fig. 1A; Table 1). NBQX (10 mg/kg) blocked the (>)-ketamine (17 mg/kg)-induced increase in spontaneously active dopamine cells at a dose that did not affect spontaneous firing on its own (Fig. 1A; Table 1). LY341495 also significantly increased the number of spontaneously active dopamine cells in the VTA (Fig. 1C; Table 1). NBQX (10 mg/kg) blocked the LY341495 (3 mg/kg)-induced increase in spontaneously active dopamine cells without directly affecting VTA.

**TABLE 1**

**In vivo electrophysiology data summary**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean ± S.E.M. (Spikes/s)</th>
<th>% of Spikes in Bursts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ketamine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.3 ± 0.18</td>
<td>5.0 ± 0.58</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>2.3 ± 0.33</td>
<td>6.0 ± 0.59</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>2.1 ± 0.29</td>
<td>6.7 ± 0.66</td>
</tr>
<tr>
<td>17 mg/kg</td>
<td>3.1 ± 0.30</td>
<td>6.3 ± 0.72</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.4 ± 0.23</td>
<td>5.1 ± 0.48</td>
</tr>
<tr>
<td>NBQX (10 mg/kg)</td>
<td>1.5 ± 0.22</td>
<td>6.3 ± 0.66</td>
</tr>
<tr>
<td>NBQX (10 mg/kg) + ketamine</td>
<td>1.6 ± 0.31</td>
<td>6.1 ± 0.46</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.2 ± 0.15</td>
<td>5.1 ± 0.62</td>
</tr>
<tr>
<td>Ketamine (17 mg/kg alone)</td>
<td>2.5 ± 0.43</td>
<td>6.6 ± 0.58</td>
</tr>
<tr>
<td><strong>LY341495</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.2 ± 0.11</td>
<td>5.4 ± 0.12</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>1.5 ± 0.29</td>
<td>7.3 ± 0.50</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>2.9 ± 0.34</td>
<td>6.8 ± 0.28</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.0 ± 0.10</td>
<td>5.4 ± 0.52</td>
</tr>
<tr>
<td>NBQX (10 mg/kg)</td>
<td>1.0 ± 0.18</td>
<td>7.0 ± 1.10</td>
</tr>
<tr>
<td>NBQX (10 mg/kg) + LY341495 (3 mg/kg)</td>
<td>0.9 ± 0.15</td>
<td>7.1 ± 1.41</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.4 ± 0.20</td>
<td>4.2 ± 0.25</td>
</tr>
<tr>
<td>LY341495 (3 mg/kg alone)</td>
<td>2.9 ± 0.42</td>
<td>6.9 ± 0.91</td>
</tr>
<tr>
<td><strong>Citalopram</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.3 ± 0.23</td>
<td>4.1 ± 0.50</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>1.3 ± 0.12</td>
<td>4.2 ± 0.54</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>0.9 ± 0.18</td>
<td>3.6 ± 0.58</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>1.6 ± 0.27</td>
<td>4.2 ± 0.56</td>
</tr>
</tbody>
</table>

- *Ketamine significantly increased the number of spontaneously active dopamine cells in the VTA (F(2,15) = 5.4, P = 0.011).
- NBQX (10 mg/kg) blocked the ketamine (17 mg/kg)-induced increase in spontaneously active dopamine cells at a dose that did not affect spontaneous firing on its own (P > 0.05).
- Ketamine significantly increased the number of spontaneously active dopamine cells in the VTA (F(1,5) = 10.93, P = 0.02).
- LY341495 significantly increased the number of spontaneously active dopamine cells in the VTA (F(2,11) = 6.81, P = 0.013).
- NBQX (10 mg/kg) blocked the LY341495 (3 mg/kg)-induced increase in spontaneously active dopamine cells at a dose that did not affect spontaneous firing on its own (P > 0.05).
- LY341495 significantly increased the number of spontaneously active dopamine cells in the VTA (F(1,4) = 21.04; P = 0.01).
- *Citalopram did not significantly affect spontaneously active dopamine cells in the VTA (P > 0.05).
- Significantly different from vehicle-treated control.
- *data were not analyzed in these independent experiments.
dopamine cell firing on its own (Fig. 1C). Neither (±)-ketamine nor LY341495 significantly affected the firing rate or bursting activity of spontaneously active dopamine neurons in the VTA (Fig. 1, B and D, respectively; all P > 0.05). To confirm that a single dose of (±)-ketamine (17 mg/kg) or a single dose of LY341495 (3 mg/kg) would be sufficient to increase the number of spontaneously active dopamine cells when tested alone (i.e., in the absence of cumulative dosing effects), separate experiments were conducted. In contrast to (±)-ketamine and LY341495, citalopram did not significantly affect VTA dopamine cell firing when administered acutely, as measured by the number of spontaneously active cells, firing rate, or bursting (Fig. 1, E and F; all P values > 0.05). Raw data and ANOVA results for electrophysiology experiments are summarized in Table 1.

**In Vivo Microdialysis.** Activation of dopaminergic circuits by (±)-ketamine or LY341495 was also evidenced by neurochemical methods, as evaluated here. (±)-Ketamine (10 mg/kg, s.c.) and LY341495 (3 and 10 mg/kg, i.p.) increased extracellular dopamine levels in the medial prefrontal cortex of rats (Fig. 2). Peak increases of 300%–400% of control were observed with ketamine or LY341495. In contrast, citalopram did not increase extracellular levels of dopamine in the prefrontal cortex at doses (10 mg/kg, s.c.) that induce a ∼700% increase in extracellular serotonin (Bymaster et al., 2002). Similarly, both (±)-ketamine and LY341495 increased extracellular dopamine levels in the nucleus accumbens of freely moving rats. Top Panel: unfilled circles=vehicle; filled circles=ketamine (25 mg/kg, i.p.). Data were analyzed by two-way ANOVA. Ketamine (25 mg/kg, i.p.): F(8, 126) = 17.39, P < 0.0001 (time); F(1, 126) = 165.3, P < 0.0001 (treatment); F(8, 126) = 12.34, P < 0.0001 (time × treatment interaction). LY341495: F(8, 378) = 8.317, P < 0.0001 (time); F(4, 378) = 4.996, P < 0.001 (treatment); F(32, 378) = 0.7542, P = 0.83 (time × treatment interaction). Each point represents the mean ± S.E.M. of recordings from five or six rats.
extracellular dopamine levels in the nucleus accumbens of rats (Fig. 3). Peak increases of 150%–175% of control were observed with (+)-ketamine or LY341495. In contrast, citalopram did not increase levels of dopamine in the nucleus accumbens at doses known to elevate extracellular serotonin levels in other areas (5 mg/kg, i.p.) (Weikop et al., 2007).

**Potentiation of Quinpirole-Induced Locomotor Activity.** In these experiments, we evaluated the expression of dopaminergic hypersensitivity engendered by these compounds on locomotor activation driven by the dopamine agonist quinpirole. Over the same dose range studied in the earlier experiments in this series, (+)-ketamine and LY341495 produced mild locomotor hyperactivity, which was generally not significantly different from vehicle control values (Fig. 4). However, when coadministered with quinpirole, both molecules significantly potentiated the locomotor-stimulating effects of quinpirole to levels greater than either drug alone (Fig. 4). In marked contrast, the locomotor stimulant efficacy of quinpirole was unchanged across a wide and high-dose range with citalopram (Fig. 4).

**Forced-Swim Test: Rats.** Ketamine has been dosed primarily by the i.v. route in depressed patients, whereas the antidepressant effects of an mGlu2/3 receptor antagonist have yet to be evaluated in patients. In the rat forced-swim assay, we compared the effects of these molecules with those of citalopram. (+)-Ketamine (i.v.) and LY341495 (i.p.) both decreased immobility times in rats under forced-swim conditions, an effect comparable to the positive control antidepressant imipramine (30 mg/kg, i.p.) (Fig. 5). In contrast, citalopram, up to 10 mg/kg p.o., did not engender significant modification of times spent immobile (Fig. 5).

**Forced-Swim Test: Mice.** The SSRI antidepressants are known to be potential false-negatives in the rat forced-swim assay (Reneric and Lucki, 1998), and that effect was confirmed here with citalopram in rats (Fig. 5). Therefore, we used NIH Swiss mice that have reliably shown antidepressant phenotypes in our laboratory with SSRI antidepressants (Li et al., 2003) to directly compare effects of citalopram with (+)-ketamine and LY341495. In these mice, both (+)-ketamine (i.p.) and LY341495 (i.p.) induced dose-dependent and large decreases in times spent immobile in the forced-swim assay (Fig. 6). Likewise, citalopram (i.p.) had activity in this assay, although the overall effect was not as large as that of either ketamine or LY341495.

Since citalopram did not increase the number of spontaneously firing dopamine neurons in the VTA (Fig. 1), we used the forced-swim test in mice to evaluate potential differential effects of AMPA receptor antagonism on comparable antidepressant-like effects of ketamine and LY341495 versus those of citalopram. Figure 6 shows that although the anti-immobility effects of (+)-ketamine and LY341495 were prevented or significantly attenuated by the AMPA receptor antagonist NBQX, the effects of citalopram were not affected by pretreatment with NBQX.

**Tail-Suspension Test in SSRI-Resistant Mice.** Prior studies by two laboratories with different apparatuses have shown a relative insensitivity of standard-of-care antidepressants (e.g., imipramine, bupropion) in a version of the tail-suspension test using the CD1 strain mouse (Witkin et al., 2011). Although (+)-ketamine and LY341495 produced large, dose-dependent reversal of immobility of CD1 mice in the tail-suspension assay, citalopram up to 10 mg/kg i.p. did not significantly alter behavior (Fig. 7).
Each point represents the mean before testing by i.v. injection (tail vein). LY341495 was given i.p., 30 minutes prior; NBQX, i.p., 60 minutes prior; and citalopram p.o., 60 minutes before testing. Analyzed by ANOVA followed by post hoc Dunnett.

Data from the present set of experiments document that enhanced dopaminergic neurotransmission in these preclinical models is associated with the actions of ketamine, a known rapidly acting antidepressant. The isomorphic effects on dopamine transmission by the mGlu2/3 receptor antagonist LY341495 points to yet another mechanistic overlap in the pharmacologic actions of NMDA receptor blockade and antagonism of mGlu2/3 receptors (Hascup et al., 2010; Koike et al., 2011; Dwyer et al., 2012). In addition to documenting this commonality in a mechanism of action of these classes of agents, our data also define the distinct mechanism of action of the SSRI antidepressant citalopram. Overall, these findings suggest that measures of dopamine neurotransmission might enable detection of rapidly acting antidepressant drugs and, in addition, that mGlu2/3 receptor antagonists might be antidepressant in patients. We demonstrate here that the NMDA receptor antagonist ketamine, which has shown rapid, robust, and sustained antidepressant efficacy in treatment-resistant depressed (TRD) patients (reviewed in Abdallah et al., 2015), potently modulates mesolimbic dopamine transmission, as demonstrated by an increase in spontaneously active dopamine cells in the VTA (see also French and Ceci, 1990), increased dopamine release in the nucleus accumbens and prefrontal cortex, and potentiated quinpirole-stimulated locomotor behavior. Consistent with the growing experimental literature on parallel mechanisms in the pharmacological actions of ketamine and mGlu2/3 receptor antagonists (Alt et al., 2006; Li et al., 2010; Dwyer et al., 2012), we show here that commonalities exist at the level of dopamine neurotransmission as well.

In contrast, such electrophysiologic, neurochemical, and behavioral effects on dopamine transmission were not observed after acute administration of the SSRI antidepressant citalopram. The data for citalopram confirm and extend previous reports that citalopram reduced burst-firing activity of VTA dopamine cells, without affecting the number or firing rate of spontaneously active dopamine cells (Dremencov et al., 2009). Similarly, although ketamine (or phencyclidine) and LY341495 enhanced extracellular concentrations of dopamine in the nucleus accumbens and prefrontal cortex in the present studies and others (Moghaddam et al., 1997; Karasawa et al., 2010), citalopram does not stimulate dopamine efflux in these brain areas (Bymaster et al., 2002). Furthermore, the effects of ketamine and LY341495 on immobility reductions in the mouse forced-swim assay were attenuated by NBQX, whereas the antidepressant-like effects of citalopram were not sensitive to AMPA receptor blockade. Thus, there are qualitative differences in the acute actions of the SSRI antidepressant citalopram versus either ketamine or LY341495.

Consistent with the hypothesis that ketamine’s efficacy in TRD patients might be mediated by the facilitation of glutamate signaling, the modulation of dopamine neurotransmission was blocked by the AMPA receptor antagonist NBQX (Maeng et al., 2008; Koike et al., 2011). We replicated and extended these results here, demonstrating that NBQX attenuated the behavioral, electrophysiologic, and neurochemical effects of ketamine and LY341495, but not citalopram. Indeed, one of the most consistent findings over the past several years linking the actions of antidepressants with glutamate has been the convergent preclinical and clinical data implicating amplification of AMPA receptor signaling in the initiation and regulation of antidepressant effects (Alt et al., 2006; Valentine and Sanacora, 2009), including the effects of ketamine (Maeng et al., 2008; Autry et al., 2011; Koike et al., 2011; Tizabi et al., 2012). Fukumoto et al. (2016) described activation of the dorsal raphe nucleus and enhanced 5-HT signaling by either systemically administered or mPFC microinjection of LY341495 or ketamine. They concluded that the primary effect of these agents is to enhance AMPA signaling in the mPFC leading to excitatory input to the DRN and enhanced 5HT outflow. It seems plausible that in an analogous way, PFC-VTA circuitry may be underpinning the rapid increase in VTA neuron firing with these agents, as shown in our studies. Recent findings have similarly suggested that Symbyax, the only FDA-approved drug for TRD, also has NMDA/AMPA receptor actions in the medial prefrontal cortex comparable to that of ketamine (Bjorkholm et al., 2015).

Ketamine, through blockade of NMDA receptors, engenders glutamate release by suppressing fast-spiking GABAergic inhibitory inputs to pyramidal cells (Moghaddam et al., 1997). Glutamate is also negatively regulated by mGlu2/3 receptors, where presynaptic mGlu2 receptors suppress glutamate release that can be relieved by blockade with molecules such as LY341495 (Hascup et al., 2010). The dense and complex innervation of midbrain dopamine neurons by glutamatergic neurons, both extrinsic and intrinsic to the VTA,
provides ample opportunity for such interactions (Cachope and Cheer, 2014; Morales and Root, 2014). Indeed, the dynamic interplay of glutamatergic and dopaminergic systems has been previously implicated in the sensitization of dopamine pathways (Li et al., 1997).

The data discussed so far have established an association between the activation of dopaminergic neurotransmission and other antidepressant-related effects of acutely administered ketamine and LY341495, but not that of citalopram. However, causal implications of these effects on dopamine have not been established. Circumstantial support for the dopamine hypothesis comes from the convergent evidence mechanistically implicating dopamine in antidepressant effect (see Introduction). The dissociation of effects of ketamine and LY341495 from those of citalopram shown here provides another potential thread of support. The most compelling data come from recent elegant work on the relationship of VTA dopamine neurons and depression-related behaviors. By optogenetic control of dopamine neurons in the VTA, inhibition of these cells induced a depression-like behavioral phenotype in mice (Tye et al., 2013). Moreover, when comparable depression-like behaviors were engendered by chronic mild stress, these behaviors could be reversed by phasic activation of VTA neurons and modified neural encoding in the NAc. A complementary set of studies independently and simultaneously identified VTA neuron firing patterns as key

Fig. 6. Left: (±)-Ketamine, LY341495, as well as citalopram decreased the time spent immobile in the forced-swim assay in mice. Ketamine: \( F(3, 28) = 7.466, P < 0.001 \); LY341495: \( F(3, 44) = 14.24, P < 0.0001 \); citalopram: \( F(3, 16) = 16.72, P < 0.0001 \). Right: NBQX (10 mg/kg) attenuated these effects. Ketamine: \( F(3, 28) = 11.36, P < 0.0001 \); LY341495: \( F(3, 28) = 5.183, P < 0.01 \). All compounds were administered i.p., 30 minutes before testing except NBQX (60 minutes). Data were analyzed by ANOVA followed by post hoc Dunnett’s test. Each bar represents the mean ± S.E.M. of recordings from 4–16 mice. *Significantly different from vehicle-treated control (\( P < 0.05 \)).
to antidepressant activity (Chaudhury et al., 2013). Furthermore, in like effects of ketamine was connected by data from recent studies. In this work, learned helplessness in rats engendered by inescapable noxious stimuli impaired normal avoidance rats rendered helpless, LTP in the ventral subiculum-to-nucleus accumbens circuit, a known stress-regulating pathway (Herman and Mueller, 2006) was disrupted; this decrease in LTP was reversed by ketamine, and such restoration was dependent on dopamine D1 receptor stimulation in the NAc (Belujon and Grace, 2014). Newer potential antidepressant mechanisms have also been suggested to have a more rapid onset of action than those of SSRI antidepressants. For example, blockade of serotonin 5-HT2C receptors was recently reported to have a faster onset of action in preclinical models than citalopram; these actions were experimentally attributed to the increase in dopamine neurotransmission by this mechanism (Opal et al., 2013).

Data also exist showing that dopamine receptor antagonists can modulate synaptic depression in vivo (Kamiyama et al., 2011), behavioral effects of ketamine in the forced-swim assay (Li et al., 2015), and in preclinical models predicting subjective effects of NMDA receptor blockade (Beardsley and Balster, 1988). It must be remembered, however, that the dopaminergic hypothesis as a guide to the clinical effects of ketamine and related drugs has not been not fully realized (e.g., in the area of schizophrenia) (Moghaddam and Krystal, 2012). Dopamine antagonists do not block the subjective high induced by ketamine (Krystal et al., 1999), nor do they appear to dampen the antidepressant effects of ketamine in patients. Anecdotal reports indicate that neither haloperidol nor other antipsychotic drugs block the antidepressant effects of ketamine (C. Zarate, personal communication), whereas sedation is increased as previously reported (Krystal et al., 1999). Furthermore, in the preclinical model studied here (tail-suspension test in CD1 mice), bupropion, a DAT inhibitor in vivo in preclinical doses, was without significant effect. Therefore, for the present, the conceptualization that rapid dopaminergic facilitation might be a triggering mechanism for rapid antidepressant relief must be confirmed by additional data.

Although recognized for many years, current trends at thinking in the behavioral sciences are moving away from the idea that behavioral disorders are unitary disease entities. With MDD too, behavioral endophenotypes have been operationalized as the primary subject matter for biologic focus. Conventional antidepressants, even when effective in treating mood, do not necessarily affect motivational components of behavior. The lingering anhedonia (Argyropoulos and Nutt, 2013), although not fully understood, has a firm basis in the neurobiology of reinforced behavior, as originally elucidated by studies on the so-called reward circuits of the brain (Olds and Milner, 1954). Important for present purposes, these circuits are known to overlap and interact with those affected in MDD and those influenced by antidepressants. Dopamine neurons originating in the VTA and innervating the NAc, amygdala, and other limbic structures with projections to the cortex, where they interact with glutamatergic control circuits, provide the brain with feed forward and regulatory control of motivation, evaluation of the salience of environmental events, emotional memory, and cognitive/emotional interpretation of the world. Dopamine neurons thus are the first likely candidate for control of anhedonia. The data from Belujon and Grace (2014) in learned helplessness experiments exemplify the fact that ketamine, through its modulation of hippocampal function (i.e., LTP), might relieve anhedonia. Recently, two clinical reports with ketamine have affirmed the rapid actions of ketamine in relieving anhedonia in both bipolar disorder (Lally...
et al., 2014) and in MDD (Lally et al., 2015). These data held true even when controlling for the effects of ketamine on mood.

Conclusions

From convergent levels of analysis, the data of the present study indicate that the rapidly acting antidepressant ketamine rapidly engages and facilitates dopamine transmission. The commonalities in the data from these multiple assays between ketamine and the mGlu2/3 receptor antagonist LY341495 further document the potential for mGlu2/3 receptors to serve as a target for TRD patients. We acknowledge that the manner in which dopaminergic neurotransmission impinges on antidepressant effects is still not fully understood (c.f., Abbasova et al., 2013; Bhagwagar et al., 2015). Nonetheless, alterations in dopaminergic neurotransmission after acute treatment with novel agents, such as the mGlu2/3 receptor antagonist studied here, might serve a predictive function for a highly sought novel class of antidepressants with large effect size, rapid onset of action, and efficacy in TRD patients. Novel mGlu2/3 receptor antagonists with properties conducive to i.v. testing of this hypothesis in patients have recently been characterized (Witkin et al., 2016) and await clinical proof of concept studies.

Authorship Contributions

Participated in research design: Witkin, Li, Mitchell, Carter, Johnson, Rasmussen, Rorick-Kehn.

Conducted experiments: Witkin, Li, Overshiner, Mitchell, Carter, Johnson, Rorick-Kehn.

Performed data analysis or contributed to data interpretation: Witkin, Monn, Schoepf, Li, Overshiner, Mitchell, Carter, Johnson, Rasmussen, Rorick-Kehn.

Wrote or contributed to the writing of the manuscript: Witkin, Monn, Schoepf, Li, Mitchell, Rasmussen, Rorick-Kehn.

References


**Address correspondence to:** Dr. Jeffrey M. Witkin, Lilly Research Laboratories; Lilly Corporate Center, Indianapolis, IN 46285-0510. E-mail: jwitkin@lilly.com